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## **Novel electroporation System for both Gram-negative and Gram-positive Bacteria Assisted by Multi-Walled Carbon Nanotubes**

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### **ABSTRACT**

Gram positive (*Lactococcus lactis*) and Gram negative bacteria (*Escherichia coli*) were used to study the interaction of water-dispersible multi-walled carbon nanotubes (CNTs) with the bacterial cell envelope during microwave (MW) energy exposure. It was observed that the addition of a tiny amount of CNTs to a medium containing bacteria and subsequent exposure of the samples to MW, leads to an intimate contact between the CNT tips and the cell envelope. This phenomenon can be explained in terms of attractive forces between opposite charges of polar structures. Since CNTs under MW irradiation behave like electric dipoles, this would make it possible for the CNTs to target the cell surface without inducing changes in the cell shape and viability. Thus, the electrochemical properties of CNTs and their capillarity make them useful tools for cell manipulation, and therefore for the intracellular transport of drugs, dyes or biomolecules.

### **INTRODUCTION**

Electroporation is a technique in which bio-membranes are permeabilized by pulsed electric fields of several kV/cm amplitude and submicrosecond duration. Thereby transient pores via membrane disruptions are formed and solvated molecules, like DNA, or drugs can be transferred into living cells. However, existing electroporation technology is limited by its ability to treat large quantities of cell material and DNA [1]. Additionally, the application of high electric field pulses can lead to irreversible electroporation and, consequently, cell lysis.

To overcome these limitations, miniaturized protocols for electroporation by localized electroporeabilization have been developed. These new techniques include, among others, electrolyte filled capillaries [1,2], micropipettes [3] and chip structures [4]. Unlike bulk electroporation, these techniques not only reduce the voltage applied (a few volts versus kilovolts), but also the amount of cell material and agents considerably, and make it possible to electroporate a single cell [1,4]. Though these techniques are well suited for treating single mammalian cells, they are not suited for targeting bacteria and microorganisms. Moreover, these methods are difficult to use in areas where the cell surface is hard, such as plant cells and bacterial cellwalls or capsules.

Further miniaturization of the electroporation systems to the nanoscale will allow selective manipulation of cell organelles in eukaryotic cells and prokaryotic microorganisms. The main goal of this research is to scale down the large scale of cell targeting by the use of CNTs as potential "electroporation devices".

## EXPERIMENTAL DETAILS

### Bacterial strain and cultivation

The widely used *Escherichia coli* (*E. coli*) strain DH5 $\alpha$  and *Lactococcus lactis* (*L. lactis*) strain DSMZ 20481 were assayed for their ability to interact with CNTs. *E. coli* was cultivated overnight in LB broth. *L. lactis* subsp. *Lactis* strain DSM 4366 was cultivated in medium 449: M17 for *Lactic Streptococci*. Cells in log phase growth were used throughout.

### Multi-walled carbon nanotubes (CNTs)

CNTs were studied for their ability to interact with the cell membrane surface of bacteria. The process starts from perpendicularly aligned CNTs, which were provided by Nanolab Inc (Fig 1A) and were grown by chemical vapor deposition (PCVD) [5] on a silicon substrate using Ni as a catalyst. Stable dispersions of CNT have been produced using mild bath sonication and subsequent dispersion into water with the aid of a strong acid treatment (Figure 1B). Thus, CNTs were functionalized in an acid solution (12 h in a nitric/sulfuric (1:3) acid solution) by means of an oxidation process, which generates carboxylic groups at the ends and in the defects of the sidewalls of the CNTs. Chemically processed and sonicated CNTs are in the range of hundreds of microns.

### Gold nanoparticles

Silica-coated gold nanoparticles shown in figure 1C, were prepared as previously described [6,7] and concentrated by centrifugation, which also allowed removal of the excess sodium silicate.

### Bacterial cells dispersed in buffer solution containing hydrophilic CNTs, and subsequent exposure to microwave irradiation

In order to prepare bacterial suspensions, 10 ml cells from an early log culture ( $7-9 \times 10^6$ /ml) were removed by centrifugation ( $4000 \times g$ , for 5 min), washed twice with PBS Dulbecco (1X) (Biochrom) buffer, and then, redispersed in 4 ml PBS buffer. A tiny amount of water dispersible CNT (10  $\mu$ l) was added to the cell suspension. The resulting coarse pre-dispersion was homogenized using a vortex mixer at low speed. The dispersion was separated into 2 ml fractions and chilled on ice for 20 min. One fraction was then exposed to microwave irradiation (MW; Magnetron 100 W, 2.45 GHz for 1 sec), the other was prepared as a control. The same procedure was repeated for different MW exposure times (2-10 sec.). MW-irradiated bacterial samples were then filtrated through sterile membranes (Sartorius, pore size 5  $\mu$ m). After this step, the filtered bacterial suspension was centrifuged ( $4000 \times g$ , for 5 min and the supernatant discarded. Growth of viable cells was controlled after microwave exposure. A very small amount of silica-coated gold nanoparticles (2  $\mu$ l) was added to *L. lactis* cells already dispersed in buffer solution containing CNTs. The MW treatment and rinsing of the cells is analogous to the procedure that we use for *E. coli* cells. Samples were taken for TEM examination.

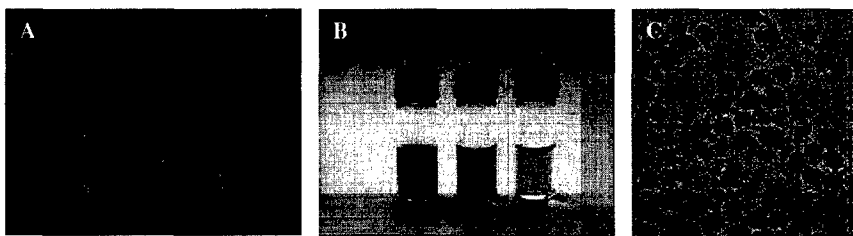
### TEM Examinations

Samples for transmission electron microscopy (TEM) analysis were deposited on carbon-coated nickel TEM grids (Plano). TEM measurements were performed on a Leo instrument operated at an accelerating voltage at 120 kV.

## RESULTS

### Bacterial viability in the presence of CNTs after MW exposure

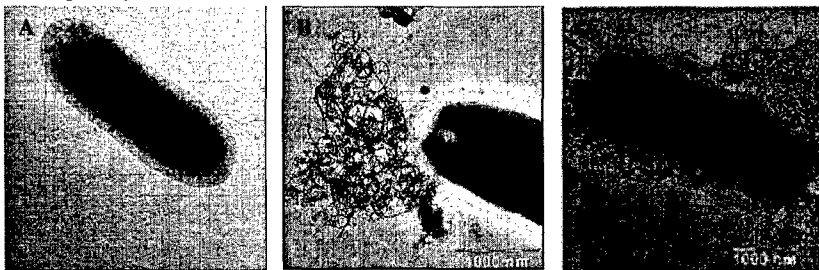
Cell viability after 1-10 sec MW exposure was determined by transfer of samples (innoculum) into a fresh culture medium. The results of cell viability and cell growth provide insight into the thermal stability of cells, and the ability of these cells to withstand a tiny amount of water dispersible CNTs (see Figure 1B). After an overnight incubation, only bacteria (*E. coli* and *L. lactis*) coming from MW exposure duration between 1 -5 sec MW were viable. Cells coming from samples exposed more than 6 sec. were not able to grow in fresh broth medium. In view of these results, all experiments were performed under a MW exposure of 4 seconds. The growth of bacteria after 4 sec. exposure time was slightly reduced compared with untreated control cells (data not shown).



**Figure 1.** A) SEM image of vertical aligned CNTs prior to surface oxidation and sonication. B) Water dispersible CNTs in three different concentration. C) TEM image of silica-coated gold nanoparticles average size 10 nm.

### TEM- studies concerning the interaction of bacterial cells with CNTs

When added at random to bacteria, the CNTs are expected to remain tangled within bacterial suspension after mixing condition. However, *E. coli* cells (Figure 2A) show a great affinity for CNTs (Figure 2B). The "hairy" appearance of the cells under the TEM microscope seems to be due to an electrostatic interaction between the CNTs and the cell surface. When exposed to microwave (4 sec) a sufficiently high concentration of the CNTs covering the cell surface locally reorient perpendicular to the direction of the cell surface, and look like "needles pricking the cells". It seems that the CNTs are embedded in the cell envelope (Figure 2C).



**Figure 2.** TEM images of A) Typical morphology of *E. coli* log-phase cells growing in LB Broth. B) *E. coli* cell treated with CNTs prior to MW and C) *E. coli* cells pretreated with CNTs after 4 sec. MW exposure.

### The interaction of CNTs and gold nanoparticles with *L. lactis* bacteria

Gram positive *L. lactis* (Figure 3A) bacterium shows a quite different interaction pattern with CNTs and gold nanoparticles. TEM examinations show that before being exposed to MW *L. lactis* cells do not attract a significant number of CNTs or gold nanoparticles in their vicinity, as seen by *E. coli* (Figure 2B). After exposure to MW irradiation (4 sec), however, *L. lactis* cells show a strong affinity to CNTs. However, unlike the CNTs bound to the cell, gold nanoparticles tend to accumulate at the surface of CNTs. Only scarce gold nanoparticles are visible at the cellular surface. Increasing the MW exposure (6 sec), it was possible to see more gold as discrete particles interacting with the cell surface (Figure 3B).



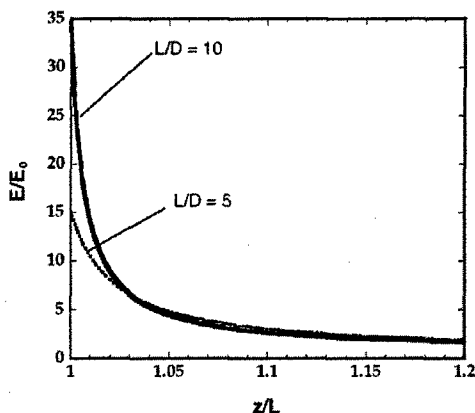
**Figure 3.** TEM images of *L. lactis* cells interacting with CNTs and silica-coated gold nanoparticles A) Typical morphology of *L. lactis* cells prior to MW exposure. B) *L. lactis* cells after 4 sec MW exposure. C) *L. lactis* cells after 6 sec. MW exposure. Both CNTs and gold nanoparticles are observed as discrete structures on the cell surface.

### DISCUSSION

Carbon nanotubes have unique properties that lend themselves to many potential applications. In order for nanotubes to reach their full potential, it is necessary to have a comprehensive understanding of their electronic properties. However, acquiring this knowledge presents major challenges. A nanotube's thermal, electrical, and physical properties vary significantly with its diameter and chirality [8].

During the MW irradiation process dipoles formed on the nanotube surface and interstitial channels interact with the microwave field leading to some absorption, which is lost through heating [9]. The activation of carbon nanotubes (CNTs) is due essentially to the "lightening rod" effect, and occurs when a metallic rod (e.g., a multi-walled CNT) is inserted into a region of a uniform electric field  $E_0$ . This causes a strong field enhancement at the CNT tip. A simple estimate of this enhancement for a single, straight CNT is given by  $E/E_0 = \alpha L/D$ , where  $\alpha \sim 3$  is a constant,  $E$  is the field at the tip,  $L$  is the CNT length, and  $D$  is its diameter. Thus, the larger the aspect ratio ( $L/D$ ) of a CNT, the larger the field enhancement at the tip. The same effect is responsible for the lowest observed threshold of  $< 1 \text{ V}/\mu\text{m}$  for the electron field emission from CNT [10]. By modeling a nanotube as a prolate, metallic spheroid of length  $L$  and width  $D$ , we can calculate the field enhancement more accurately [11]. Figure 4 shows the electric field along the axis of a CNT calculated for two CNTs with the aspect ratio  $L/D = 5$  and 10. The tip of the nanotube is at  $z/L = 1$ . It is clear, that while the CNT with the aspect ratio 5 enhances the field at the tip by a factor of 15, the tube with the aspect ratio 10

more than doubles that value to 35. Even though these calculations are static, they apply to the microwave fields as well, because the plasma frequencies of both CNTs and gold nanoparticles are much higher (UV range) than the microwave frequencies [12].



**Figure 4.** Calculated field enhancement at the tip of a CNT simulated as an oblate spheroid with the aspect  $L/D$

#### Transmembrane pores induced by activated CNTs?

Since dipoles both on the surface and interstitial channels of nanotubes are involved in the activation of single wall CNTs [9], the interfacial mechanism for CNT-cell targeting was investigated. In this study, we observed that CNTs under MW exposure can target bacterial cells. Although *E. coli* cells spontaneously attract CNTs to their cell surface, mostly randomly oriented, several aspects of the MW-induced oriented assembly of CNT-*E. coli* cell surface indicate that CNTs might create openings in the plasma membrane like those associated with bulk electroporation.

TEM images show that after MW exposure, CNTs are disposed in such a way that, in the plane perpendicular to the CNT long axis, the cell envelope seems to be actually anchoring single CNTs. Thus, individual CNTs contacting the bacterial cell via their end tips seems to be an important feature of nanoscale cell targeting. Phenomena due to primary electrostatic interaction between CNTs and cell envelope components and secondary processes such as energy transfer (heating) and chemical exchange are involved in this interfacial interaction. Unlike *E. coli*, *L. lactis* prior to MW exposure did not show any significant nanostructure attached to their surface (see Figure 3A). It seems that the *L. Lactis* cell walls having a distinctly thick layer of peptidoglycan (which contrasts with the thin layer in *E. coli*) play an important role in determining the use of MW irradiation for CNT-assisted cell targeting and possible transmembrane transport of gold nanoparticles .

MW activated CNTs operating through the so-called "end tip" mode of action would have a greater potential for membrane discrimination, if the end tips are functionalised, thus creating molecular probes (resistant to proteasas) with potential applications in cell biology and biomedicine [12,13]. Further, this discovery allows us to explore their potential application as nanodevices for electroporation. This latter effect must consider the area

around a charged CNT. The electric field around the CNT end tip can exert a force on any charged object in its vicinity. The closer the charged object is brought to the charged object creating the field, the greater the force exerted on it. As a result, local points at the cell envelope supporting a transient current undergo structural rearrangements of cell envelope components leading to the onset of nanopores. Further TEM studies with cross section are needed to clarify both the possible transmembrane location of CNT after MW exposure and the intracellular transport of gold nanoparticles. Nevertheless, it was possible to observe a relationship between MW irradiation and MWCNT-cell interaction. The latter phenomenon was especially evident when *L. lactis* as a gram positive bacteria were targeted with CNT and gold nanoparticles.

## CONCLUSIONS

The individuality of water dispersible CNTs and their electrochemical properties enable nanoscale cell targeting. In this study, the effect of MW-activated CNTs and simultaneous transport of gold nanoparticles across the cell envelope was not detrimental to cell viability, thus, CNTs can be considered as potential devices for nanoscale manipulation of cells. It may be possible to design CNTs to recognize cell surfaces; if one could achieve that goal, then it may be possible to deliver molecules/particles confined in their interstitial channels to a particular cell type. Many application areas can be envisioned in cell biology and biomedicine, for instance, single cell electroporation, biosensors, drug delivery, and cell repair.

## ACKNOWLEDGMENTS

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